ANTIGEN-INDUCED CHANGES IN LYMPHOID CELL HISTONES

III. In Vitro and in Extract

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ABSTRACT

Previous papers in this series have reported an acute, transitory effect of antigens on lymphoid cell nuclei. In the previous reports the effect was related to a change in ammoniacal silver (A–S) stainability of smears and cryostat sections. The variable substrate was identified as histone. This paper reports the results of an extended series of studies of histone and chromatin extracts from thymus glands exposed to antigen in vivo and in vitro. The antigen effect on A–S stainability is demonstrable not only in vitro but also in chromatin fibers representing a DNA–histone complex. However, it is not demonstrable in isolated histone fractions. The inference is drawn that the antigen-induced alteration in A–S stainability is brought about not by any quantitative change in histone, but by a biologically significant shift in histone binding, perhaps to DNA. It is suggested that alteration in DNA–histone binding during gene activation may alter A–S stainability of histones.

INTRODUCTION

We have previously reported that the injection of an antigen causes an acute transitory change in ammoniacal silver (A–S) stainability of lymphoid cell nuclei (1, 2). We have also observed that the nuclei of different cell types are stained characteristic colors by A–S and that the A–S staining of cancer cell nuclei tends to be characteristically indistinct, save for the mitotic figures. It is also noteworthy that during early embryonic development the A–S stainability is much reduced; organogenesis is virtually complete before A–S stainability becomes prominent and the colors characteristic of mature cells develop after staining. We have also observed that A–S staining discriminates between different histone fractions, when isolated and spotted on paper, by imparting characteristic colors to each (3). Thus our continuing experience with A–S staining seems to affirm the ability of the procedure to disclose histone variations in different cell types and in different functional states.

The antigen-induced change in the A–S staining of lymphoid cells appears to us to provide a particularly suitable system for probing the biological significance of histones since it uses a physiological stimulus with a high degree of specificity for the cells investigated. Accordingly we have extended our observations to include the in vitro interaction of lymphoid tissue with antigen and have compared the properties of histone and chromatin extracts of control and antigen-affected thymus glands.

MATERIALS AND METHODS

Thymus glands of CF-1 mice (Carworth Farms, Inc., New City, N. Y.) weighing 18–20 g and of 21-day-

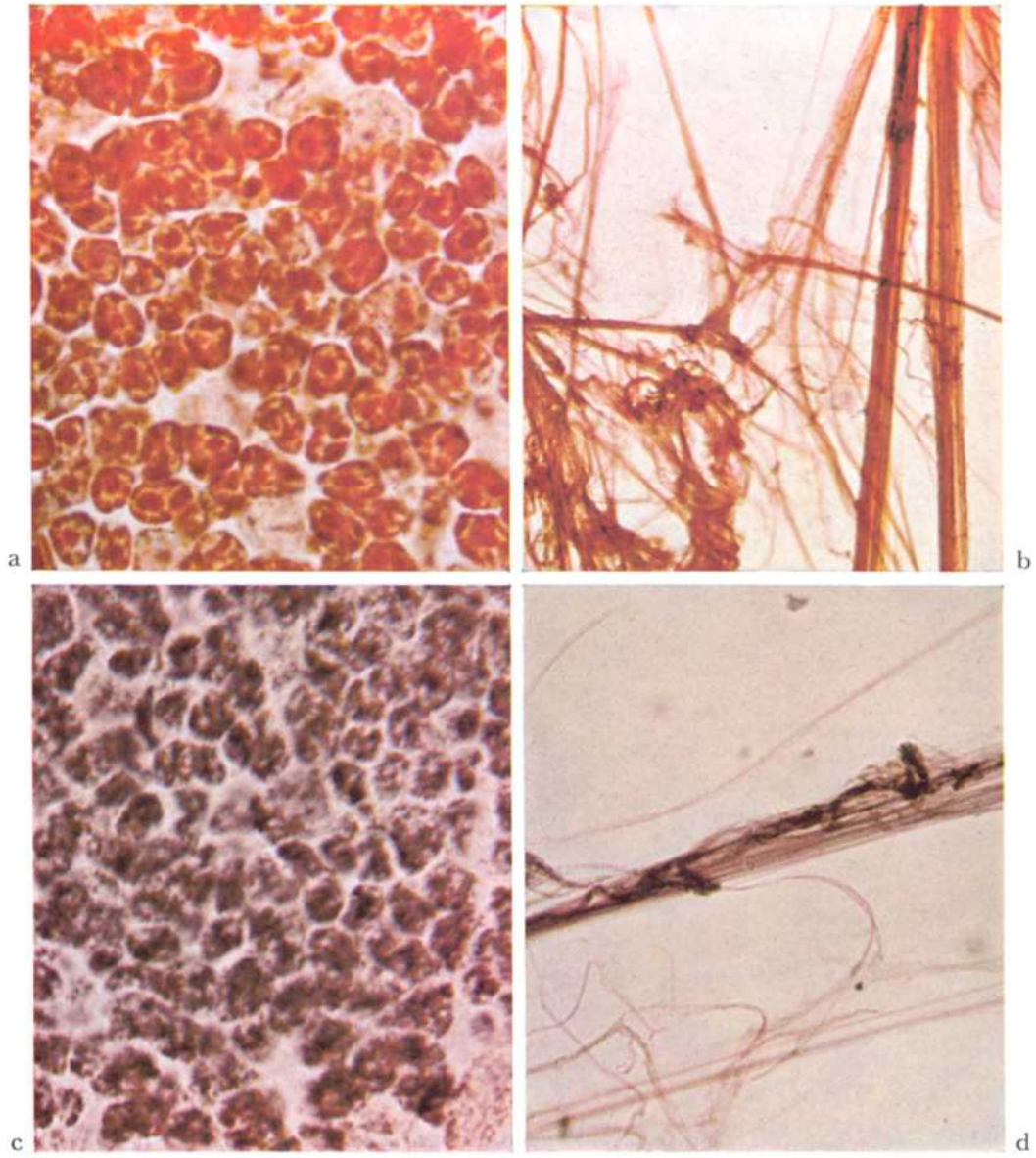

FIGURE 1 A–S staining of normal and antigen-affected thymocytes is shown together with corresponding DNP fibers. Fig. 1 *a*, cryostat section of normal thymus gland and Fig. 1 *b*, DNP fibers from same. Fig. 1 *c*, cryostat section of thymus gland exposed in vitro to antigen, and Fig. 1 *d*, DNP fibers from same, showing altered A–S staining. In addition to staining differences the DNP from control and antigen-affected thymus glands also exhibit solubility differences (11).

old weanling Lewis rats were the subject of study. Matched groups of antigen-treated and untreated animals were employed in each run of all the experiments. Each matched group consisted of three to four animals. While goat serum gamma globulin and tuberculoprotein were used as the antigen in some experiments, tetanus toxoid was used most of the time.

In vivo antigen administration was in the form of intraperitoneal injection of 0.1 ml of tetanus toxoid (Wyeth Laboratories, Marietta, Pa.), or antigen equivalent, 2 hr before sacrifice.

In vitro antigen administration was as follows. Separate lobes of freshly excised thymus glands were placed in vials containing antigen in saline, e.g.

2 ml of 0.9% sodium chloride plus 0.2 ml of a 1:10 dilution of antigen. At the same time an equal number of thymus lobes was placed in a vial containing only the saline solution. The vials were incubated at 37°C for 2 hr on a vibrator. At the end of this period the glands were rinsed in cold saline and extracted as follows.

Extraction Procedures

HISTONES: The glands were homogenized and extracted in cold (0–4°C) 5% perchloric acid (4 ml per three thymus glands) for 1 hr. The homogenate was then centrifuged in a refrigerated centrifuge at 17,000 *g* for 15 min. The supernate was decanted and the protein was precipitated by adding 100% trichloroacetic acid (w/v) to yield a final concentration of 20%. The precipitate was collected by centrifugation, washed twice with acetone, and then dissolved in 0.25 N HCl. This perchloric soluble fraction was termed 1B. The perchloric extracted cellular residues were resuspended in cold 0.50 N HCl and extracted for 1 hr in the cold. The mixture was then centrifuged, and the supernate was collected and precipitated as before, then dissolved in 0.25 N HCl, and designated extract 2B. The remaining cellular residues were resuspended in 1.0 N HCl and extracted overnight in the cold. The mixture was then centrifuged, and the supernate was collected and treated as before to yield extract 3B.

DEOXYNUCLEOPROTEIN (DNP): The glands were homogenized in cold (0–4°C) 2 M NaCl solution (20 ml per three glands) and then extracted with constant gentle agitation for 1 hr in the same solution. The suspension was centrifuged for 20 min in the refrigerated centrifuge at 17,000 *g*. The supernate was collected and then diluted to 0.16 M with distilled water: 12.4 ml of distilled water per 1 ml of extract. The DNP fibers that formed after dilution were collected on a glass rod, rinsed in 0.16 M NaCl solution, and then dissolved in 8–10 ml of 2 M NaCl. The solution was then centrifuged and a clear supernate was obtained. From this DNP solution fiber samples were prepared by adding aliquots of the 2 M solution to large excesses of 0.16 M NaCl solutions, i.e., 0.2 ml of DNP solution to 150 ml of the dilute salt solution. The fibers thus formed were taken up on glass microscope slides and fixed while wet in 10% acetate-neutralized formalin (2 g sodium acetate per 100 ml of 10% formalin).

Histone was also extracted from the isolated DNP fibers in 0.25 N HCl. This extract was termed DNP-H.

The results reported below represent data obtained from not less than 15 mice or rats for each type of study.

Before extraction, samples of the thymus glands were taken for cryostat sectioning in order to compare the effect of the antigen on the whole gland, i.e., source of material, with the effect on the derived extracts in terms of A–S staining.

The procedure for A–S staining was as given in previous reports (1), except that an aqueous mount (glycogel) was used. All steps in the procedure were controlled by conjoint treatment of antigen-exposed and nonantigen-exposed material. A–S color was compared by inspection, supplemented routinely by spectral determinations with a Canalco (Bethesda, Md.) microspectrophotometer. In practice, a complete spectrum was not necessary since we have found that the 640/460 mμ ratio serves to distinguish all gradations of A–S color.

The effect of prolonged fixation on A–S color was also investigated, namely by holding the specimens in 10% acetate-neutralized formalin at 28°C for 2 days to 2 wk.

Ultraviolet spectra of the DNP extracts were measured on a Beckman DU spectrometer. The ratio of 230/260 mμ was taken as an empirical approximation of the protein to nucleic acid, in accordance with the procedure of Mirsky and Pollister (4).

The relationship of total protein to DNA of individual nuclei (cryostat sections) and of DNP fibers mounted on glass slides was studied from Feulgen preparations counterstained with naphthol yellow, as in the method of Deitch (5). A practical representation of the relative contributions of the two dyes is the 440/560 mμ ratio.

Paper spots of the histone extracts were prepared by allowing a drop to flow from a disposable capillary pipette onto oxoid cellulose acetate paper (Consolidated Laboratories Inc., Chicago, Ill.). The spots were air-dried, fixed, and stained with A–S in a Petri dish in the usual way. This method of preparing histone spots has been found to yield a reproducible type of spreading with similar extracts. Furthermore, the present study was concerned with the color of the A–S staining which is unchanged by varying the size of spots produced from the same extract.

The histone extracts, 1B, 2B, 3B, and DNP-H, were further fractionated by means of disc electrophoresis. The extracts were run on the standard low pH polyacrylamide gel of Ornstein and Davis (6) in 0.37 M glycine buffered to pH 4.0 with acetic acid. The bands were visualized with amidoschwarz. Lowry protein determinations on these fractions were also run for us by Dr. William Burke of this department.

In order to provide an additional control for the studies on the in vitro effects of antigens on the A–S staining of thymocytes and thymic DNP, we have performed similar studies on rat kidneys.

RESULTS

A–S Staining

In vitro exposure to antigen altered the A–S staining of thymocyte nuclei of excised thymus glands (cryostat sections) in a fashion similar to that previously reported for in vivo administration of antigen. Thus thymus glands incubated as controls in saline alone stain the characteristic yellow color of normal thymus tissue, whereas glands exposed to antigen in vitro stain a more brownish black. The present study also discloses that DNP fibers stain like the nuclei from which they were obtained. This latter observation is depicted in Fig. 1. Cytophotometric measurements of the color of the nuclei and the fibers are presented in Table I. It is apparent that antigen induces a change in A–S staining after exposure in vitro as well as in vivo, and that the altered stainability persists in isolated chromatin fibers.

TABLE I

640/460 mμ ratio of A–S stained thymus glands. Control, C; antigen-exposed, T.

		Time in formalin		
		1 hr	24 hr	72 hr
Cryostat sections	C	0.43	0.51	0.51
	T	0.72	0.60	0.51
DNP	C	0.20	0.24	0.25
	T	0.45	0.36	0.26

These findings apply to the studies of the thymus glands of rat and mouse alike, and to the use of goat serum and tuberculin as well as tetanus toxoid. On the other hand, no antigen effect was demonstrable in kidney sections or in DNP fibers prepared from such kidneys.

The A–S staining of histones extracted in dilute acid from antigen-exposed thymus glands is not distinguishable from the A–S staining of histones from control thymus glands. As indicated before (3), various histone fractions are stained in characteristic fashion by A–S, namely, lysine-rich histones stain yellowish while arginine-rich histones tend to stain blackish. In the present study such differences still obtain. Thus fractions 1B and 2B, which contain appreciable amounts of lysine-rich histone, stain yellowish, whereas fraction 3B, which is predominantly arginine-rich, stains gray. While such differences as these obtained, no consistent change in the antigen-treated material is found for any of the fractions, including 1B, 2B, 3B, and DNP-H. Fig. 2 depicts representative disc electrophoresis gel columns of each of these fractions. Antigen-exposed extracts present no consistent difference. Here, as in all the histone fractions, the band pattern is characteristic of the fraction and indistinguishable in the test material.

Protein-to-DNA Relationship

UV ABSORPTION: Measurement of DNP extracts in 2 M NaCl failed to reveal any significant difference in the test material (see Table II).

Feulgen–Naphthol Yellow Staining. The relative intensity of naphthol yellow over Feulgen staining is not demonstrably altered by exposure to antigen either in thymocyte nuclei or DNP fibers isolated from such nuclei. See Table II.

Lowry protein-determinations of the various histone extracts show no consistent difference in the amounts of histone extracted directly from control and antigen-exposed glands. The amounts of histone extracted from the DNP from control and antigen-treated glands were also similar.

In summary, then, it appears that the readily demonstrable antigen-induced change in A–S staining of thymocyte chromatin is not associated with any detectable, corresponding alteration in the amount of nuclear protein as determined by these other techniques.

PROLONGED FIXATION: It was previously reported that histone staining with A–S depends upon prior formalin "fixation." The effect of increasing times in formalin was investigated, with several pertinent results. Prolonged exposure to formalin does not alter the specificity of the stain; if anything, the nonhistone background becomes cleaner. Cytoplasmic and extracellular substances do not become A–S positive even after exposure to formalin for as long as 2 wk. However, the antigen-induced change in A–S staining becomes less pronounced with increasing times of exposure to the formalin. Thus after 72 hr of pretreatment in formalin the A–S staining of antigen-affected material becomes indistinguishable from that of the control. See Table I.

The ability of DNA to alter the A–S staining of histone was tested on artificial complexes formed between commercial fish DNA and calf thymus histone. The precipitate thus formed stains dark brown to black instead of the yellowish stain of

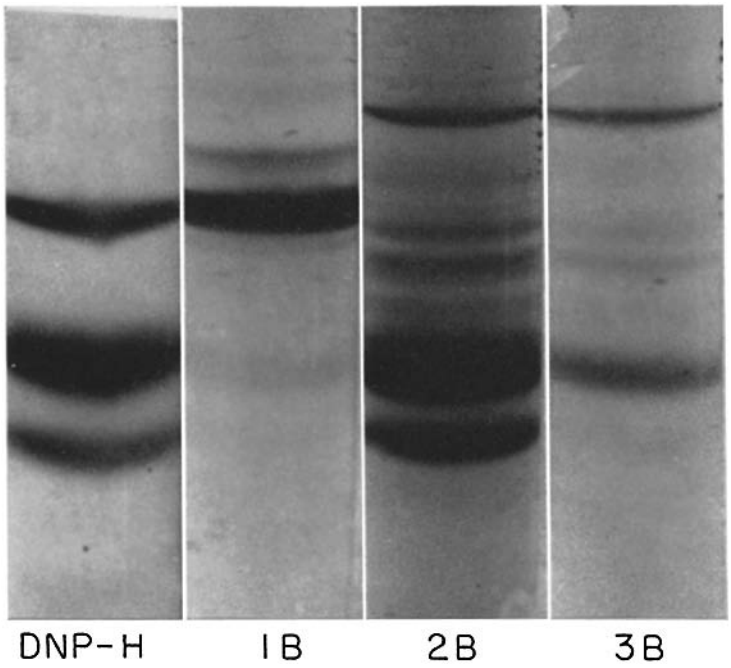

FIGURE 2 Disc electrophoresis columns showing banding patterns of lymphoid cell histone isolates. DNP-H represents a dilute acid extract of DNP fibers, while 1B, 2B and 3B represent extracts of lymphoid cell homogenates. Identical bands are obtained from control and antigen-affected thymus glands.

TABLE II
Protein/DNA relationship in thymus nuclei and DNP fibers

	2M extract Beckman 230/260 mμ	Naphthol yellow/Feulgen	
		Cryostat sections	DNP fibers
C	0.83	1.18	0.86
T	0.78	1.22	0.88

the isolated histone, while the DNA alone does not stain with A–S.

COMMENTS

An extended series of studies has confirmed our earlier reports of a change in the A–S staining of lymphoid cell nuclei shortly after the injection of antigen. The case has been extended to include different species of animals and various kinds of antigen. More importantly it has been shown that the effect may be induced in vitro, and also that the antigen-induced change is demonstrable in DNP fibers. The antigen-induced alteration in the DNP complex is also evident in terms of changes in the solubility properties of DNP extracted from control and antigen-affected thymocytes. While these data will be reported in detail elsewhere, it is noteworthy that serial dilution of DNP solutions in 2 M-sodium chloride differentiates the control and test materials in terms of fiber formation (8). Despite such antigen-induced differences in the A–S staining of thymocyte nuclei and DNP fibers, differences are not evident in histones isolated from such material.

Some comment might be made regarding the extraction of histones from whole thymus glands rather than from isolated nuclei. In selected instances we have stained histones extracted from isolated thymocyte nuclei (prepared by Dr. William Burke of this department) and have found the staining to be indistinguishable from that of homologous extracts of homogenates. The same is true in regard to the preparation of DNP extracts, namely extracts of thymic homogenates with and without added EDTA and extracts of isolated thymocyte nuclei yield fibers which stain similarly with A–S. It should also be noted that

the cytoplasm of thymocytes does not contain A–S stainable material.

Our practice of washing trichloroacetic acid-precipitated histones with acetone might engender some loss of histones as reported by Laurence, et al. (9). If acetone-soluble trichloroacetates of histone included an antigen-sensitive component, it is conceivable that an actual loss would then be undetectable. In view of this possibility we have isolated such acetone-soluble fractions from control and antigen-treated thymus glands. Spots were prepared and stained with alkaline fast green and A–S. No differences were evident between the control and test material.

Furthermore, it is noteworthy that the extraction of histones from the DNP fibers (DNP-H) did not involve trichloroacetic acid precipitation and acetone washings. The DNA-bound histones from such fibers were extracted directly with HCl. Although the DNP fibers from the control and antigen-affected thymus glands stained differently with A–S, the histones extracted from such fibers were indistinguishable as judged by A–S staining and electrophoresis. Nor have we found evidence of an antigen-induced loss of DNA-bound protein as judged by UV measurements of DNP extracts and the naphthol yellow/Feulgen staining of DNP fibers and thymocyte nuclei. Finally, the fact that after prolonged formalin fixation the antigen-affected thymus glands stained like the controls speaks against an antigen-induced loss of histones from the nuclei. If no gross quantitative change has occurred in the DNA, as we know to be the case, or in the histone, then we must assume that the observed alteration in staining reflects a change in the reactive groups of histone. It is pertinent to recall that the A–S staining of histones has an obligatory requirement of prior exposure to formaldehyde. According to Frankfurt, the formaldehyde breaks histone–DNA bonds, and variations in the staining of different cell types is a reflection of the strength of such bonds (10). If this suggestion is correct, and our observations are at least consistent with this view, the antigen-induced change in A–S staining would reflect a change in the histone–DNA binding. However, it is not clear at this time whether such changes involve still other components.

Our previous studies had indicated that lysine-rich histones stain yellowish whereas arginine-rich histones stain blackish. It would seem that in control thymic DNP the ϵ-amino groups are more readily reactive than the arginine groups. The exposure to antigen appears to reverse this relationship. It is noteworthy that Allfrey and Mirsky and their colleagues have demonstrated that modifications in histone-DNA interactions "signal a change in the fine structure of chromatin" and perhaps influence template activity of the chromatin (11, 12).

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